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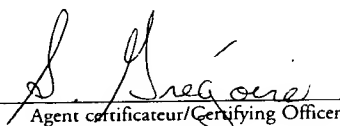
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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,267,012**, on March 25, 1999, by **UNIVERSITY OF GUELPH,** assignee of Praveen K. Saxena, Susan J. Murch and Tannis Y. Slimmon, for "Micropropagation Of Phytopharmaceutical Plants".

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ABSTRACT

The development of an *in vitro* regeneration system that utilizes a plant growth
regulator having cytokinin activity for the induction of *de novo* shoots or somatic

5   embryos on explants of phytopharmaceutical plants is provided. Transfer of the
regenerated shoots or somatic embyros into a solid or liquid medium with no plant
growth regulators results in the rapid and prolific growth of viable plantlets. The
method and i¹s modifications are intended for application to all phytopharmaceutical
plants, in particular St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin

10   (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew (*Tanacetum parthenium*), garlic
(*Allium sp.*) and the like.

Micropropagation of Phytopharmaceutical Plants

Field of the Invention

5   The present invention relates to a method for the micropropagation of phytopharmaceutical plants. In particular, there is provided a method for the *in vitro* micropropagation of St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew (*Tanacetum parthenium*), garlic (*Allium sp.*) and the like.

10

Background of the Invention

      Medicinal plants play an important role in health care throughout the world - especially in non-industrialized continents such as Africa, South America and parts of
15   Asia. Even in many industrialized countries, a number of traditional plants are widely used by a majority of people for minor to moderate everyday ailments through self-medication.

      Although many traditional medicinal plant remedies do not undergo extensive
20   scientific testing, they are very popular and their sale is not restricted by government regulatory agencies. Some medicinal plants do have substantial laboratory and clinical testing and those that fall into this category are referred to as phytopharmaceuticals.

25   One of the major problems associated with phytopharmaceutical plant preparations is the variability in the content of the medicinally active ingredients. This problem was highlighted in Belgium in 1997, when more than 100 people were diagnosed with total destruction of their kidneys through irreversible interstitial fibrosis caused by a mis-identified Chinese medicinal plant (Betz, 1998). This has led
30   to strict government controls on purity and levels of active constituents in phytopharmaceutical products in Europe. Such strict regulation does not currently exist, however, in most countries, including Canada and the United States.

The variability in medicinal content of phytopharmaceutical plants is likely the result of a variety of factors including: a) year-to-year and plant-to-plant variability in medicinal content; b) adulteration of medicinal preparations with misidentified plant

5   species; c) a lack of adequate methods for production and standardization of the crop; d) a lack of understanding of the unique plant physiology or efficacy with human consumption; and e) consumer fraud. In addition, phytopharmaceutical plant preparations, in general, are produced from field-grown crops and therefore are susceptible to infestation by bacteria, fungi and insects that can alter the medicinal

10   content of the preparations.

Past and current efforts have centered on ensuring that preparations of phytopharmaceutical plants contain the correct plant material, that the plant material is processed according to a standardized protocol and that the finished product contains

15   specific levels of a specific marker compound. Another approach has been the application of traditional pharmaceutical development methods to isolate a single "active" component and to synthesize versions of the so-called "drug". This process usually involves the dissection of the plant into chemical components and attempts to identify a single compound responsible for the induction of the desired effects in

20   mammals. Most phytopharmaceutical plant preparations are formulated from whole plants and contain a variety of compounds which may be working synergistically to produce the desired effect. Although Drug Identification Numbers (DIN) have been issued for various preparations on the basis of a standard concentration of a marker compound, there may be no physiological effect in humans which can be directly

25   attributed to this marker compound. The lack of any real knowledge base for the phytopharmaceutical industry has lead to the current situation in which the sale of phytopharmaceutical plant preparations is driven largely by enthusiasm rather than solid scientific research. Therefore, with the currently available methods, there is no way to ensure quality, efficacy or safety of phytopharmaceutical plant preparations.

30

It is essential to solve the problem of the variability in the medicinal content of preparations of phytopharmaceutical plants before consumers can use them

effectively. One such solution is the development of *in vitro* micropropagation procedures. The production of phytopharmaceutical plants *in vitro* has several advantages: a) plants are grown in sterile, standardized conditions; b) individual superior plants can be identified and clonally produced; c) plant material is consistent and therefore, precise biochemical characterizations can be achieved; and d) eventually, protocols can be developed for the improvement of the crop through genetic manipulation.

To date, there is no known general method for the *in vitro* micropropagation of phytopharmaceutical plants. There is, therefore, a need within the phytopharmaceutical plant industry for the development of such *in vitro* systems for the reliable and reproducible propagation of phytopharmaceutical plants.

Summary of the Invention:

The present invention provides an *in vitro* protocol for the mass-propagation of phytopharmaceutical plants to facilitate production of consistent plant material for industrial and research applications.

Therefore the present invention provides a method (A) for the *in vitro* micropropagation of phytopharmacuetical plants comprising:

(a) culturing a sterile explant of a phytopharmaceutical plant on a medium comprising a suitable concentration of at least one plant growth regulator having cytokinin activity for a suitable period of time to form regenerated tissue; and

(b) transferring said explant and regenerated tissue to a basal medium and culturing under standard conditions to form plantlets.

This invention is also directed to the method (A) as defined above wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew

(*Tanacetum parthenium*) and garlic (*Allium sp.*).

The invention is also directed to the method (A) as defined above wherein the plant growth regulator having cytokinin activity may be natural or synthetic and is
5   selected from thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N'-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine).

. In another of its aspects, the present invention also includes a method (B) for
10   the *in vitro* micropropagation of phytopharmaceutical plants comprising:

(a) culturing a sterile explant of a phytopharmaceutical plant on a medium comprising a suitable concentration of at least one plant growth regulator having cytokinin activity for a suitable period of time to form regenerated
15   tissue;
(b) transferring said explant and regenerated tissue to a basal medium and subculturing for a further period of time to allow optimized formation of regenerated tissue; and
(c) transferring said regenerated tissue to a basal medium and culturing under
20   standard conditions to form plantlets.

This invention is also directed to the method (B) as defined above wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew
25   (*Tanacetum parthenium*) and garlic (*Allium sp.*).

The invention is also directed to the method (B) as defined above wherein the plant growth regulator having cytokinin activity may be natural or synthetic and is selected from thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea),
30   benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N'-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine).

These and other aspects of the present invention will be described in greater detail hereinbelow.

5   Brief Description of the Drawings:

The features of the present invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

10   Figure 1 is a sketch of a bioreactor system suitable for use with the methods of the invention.

Figure 2a is a picture illustrating TDZ-induced regenerants on etiolated hypocotyl explants of St. John's wort with 10 $\mu mol \cdot L^{-1}$ TDZ. The red pigmentation at the tip of

15   St. John's wort shoot primordia, caused by the presence of the compound hypericin, is visible. (bar = 1 mm)

Figure 2b is a picture showing the development of regenerants on hypocotyl explants after 9 days of culture on TDZ-containing induction medium and 9 days of subculture

20   on basal medium. *De novo* shoots developed from all areas of the explant without an intermediate callus phase in St. John's wort. (bar = 0.75 cm)

Figure 2c is a picture showing sterile cultured plantlets grown from a single hypocotyl section after 2 months on basal medium in a Magenta box. (bar = 1.2 cm)

25

Figure 3a is a histological characterization showing *de novo* shoot induction of St. John's wort and meristematic zones formed at the hypodermal region of the hypocotyl explant (arrows) at 7 days of culture on TDZ-containing induction medium. (bar = 50 μm)

30

Figure 3b is a histological characterization showing the further development of the meristem toward the epidermis in St. John's wort hypocotyl cultured on TDZ-

containing induction medium. (bar = 50 μm)

Figure 3c is a histological characterization showing the development of shoot primordia and the development of a vascular connection (arrow) in St. John's wort hypocotyl cultured for 2 weeks on TDZ-containing induction medium. (bar = 166 μm)

Figure 3d is a histological characterization showing a well developed shoot (bar = 166 μm) and vascular connections between the adventitious shoot bud and the parental tissue (arrow) in St. John's wort hypocotyl cultured for 18 days on TDZ-containing induction medium. (bar = 166 μm)

Figure 4a is a picture illustrating callus formation and shoot regenerates appearing at the callus interface on petiole explants of *Echinacea purpurea* cultured on BAP-containing induction medium.

Figure 4b is a picture illustrating individual shoot regenerants with well defined leaf initials on petiole explants of *Echinacea purpurea* cultured on BAP-containing induction medium after 33 days of culture.

Figure 4c is a picture illustrating a late cotyledonary stage embryo on a BAP-induced petiole explant of *Echinacea purpurea*.

Figure 4d is a picture showing complete plantlets grown from embryos regenerated on petioles of *Echinacea purpurea* cultured on BAP-containing induction medium and cultured on basal medium for 2 months.

Figure 5a is a histological characterization showing periclinal cell division in the subepidermal layers of the petiole explants of *Echinacea purpurea* at day 3 of culturing on induction medium containing BAP. (bar = 20 μm)

Figure 5b is a histological characterization showing formation of promeristomatic centres (arrows) in the callus tissue of petiole explants of *Echinacea purpurea* at day 14 of culturing on induction medium containing BAP. (bar = 680 μm)

5 Figure 5c is a histological characterization showing promeristomatic centres, having cells which were smaller in size and had a dense cytoplasm and prominent nuclei (arrows), observed on the callus tissue of the petiole explants of *Echinacea purpurea* by day 14 of culturing on induction medium containing BAP. (bar = 50 μm)

10 Figure 5d is a histological characterization showing how the promeristomatic centres developed further to form dome shaped meristem zones which were well defined on the petiole explants of *Echinacea purpurea* cultured on induction medium containing BAP by day 21. (bar = 50 μm)

15 Figure 5e is a histological characterization showing development of a shoot meristem and leaf primordia on petiole explants of *Echinacea purpurea* after 21 days of culturing on induction medium containing BAP. (bar = 320 μm)

Figure 5f is a histological characterization showing a well developed shoot bud
20 surrounded by leaf primordia formed on petiole explants of *Echinacea purpurea* after 28 days of culturing on induction medium containing BAP. Note that trichomes were observed to be associated with the leaf primordia and xylem elements were present at the base of the shoot bud. (bar = 166 μm)

25 Figure 6a is a histological characterization showing a series of anticlinal and periclinal divisions leading to the formation of well defined protoderm regenerated from petiole explants of *Echinacea purpurea* at 14 days of culturing on induction medium containing BAP. (bar = 50 μm)

30 Figure 6b is a histological characterization showing a well developed heart-shaped somatic embryo with a fully formed protoderm regenerated from petiole explants of

Echinacea purpurea at 21 days of culturing on induction medium containing BAP. (bar = 320 μm)

<u>Detailed Description of the Invention:</u>

5

The present invention provides a method for the *in vitro* micropropagation phytopharmaceutical plants. As used herein, the term phytopharmaceutical plant means any medicinal plant for which some laboratory or clinical study on its efficacy or active agent identity has been done and includes such plant species as of St. John's

10 wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew (*Tanacetum parthenium*), garlic (*Allium sp.*)and the like.

In general terms, the method of the invention involves obtaining a sterile explant of a phytopharmaceutical plant and culturing it on an induction medium

15 comprising a suitable concentration of at least one plant growth regulator that has cytokinin activity for a suitable period of time. The explant could be selected from any suitable part of the plant including seeds, petiole, hypocotyl, cotyledon, stem and leaves. If the tissue is hypocotyl, it is preferably etiolated hypocotyl. The plant growth regulator having cytokinin activity may be any such synthetic or natural plant

20 growth regulator and includes thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thidiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N'-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma,gamma-dimethylallylamino purine). Preferably, the plant growth regulator having cytokinin activity is selected from thidiazuron (TDZ) and benzylaminopurine (BAP). The concentration and

25 duration of exposure of the sterile explant to the plant growth regulator(s) having cytokinin activity will vary depending on the species of the plant. A person skilled in the art can determine these values by culturing an explant from the phytopharmaceutical plant at various concentrations of the plant growth regulator(s) and for various exposure times and determining the optimal conditions for the

30 production of the maximum amount of regenerated tissue. The regenerated tissue may be in the form of shoots, calli or somatic embryos. Most commonly the regenerated tissue is in the form of shoots. It is desired to use a concentration and

duration of exposure of the plant growth regulator(s) having cytokinin activity which minimizes residual effects including a decrease in the number of regenerants, vitrification of the shoots and poor rooting of regenerants.

5   Following culture of the phytopharmaceutical plant explant on the medium comprising at least one plant growth regulator having cytokinin activity, the explant with regenerated tissue may be transferred to basal medium and cultured under standard conditions to form plantlets. Alternatively, the explant with regenerated tissues may be transferred to basal medium and subcultured for a further period of
10   time to maximize the number of regenerated tissue formed. Following this subculturing, the regenerated tissue may be transferred to either solid or liquid basal medium and cultured under standard conditions to form plantlets.

          Examples of the media which may be used for this method, which are to be
15   considered non-limiting, are presented in Table 1:

Table 1

	Induction Medium	Solid Basal	Liquid Basal
pH	5.7	5.8	5.8
MS salts (Murashige & Skoog, 1962)	+	+	+
B5 Vitamins (Gamborg, 1968)	+	+	+
Sucrose	3%	3%	3%
Gellan Gum	0.3%	0.3%	---
Growth Regulator	Cytokinin activity	---	---

20   Sterile explants of phytopharmaceutical plants may be obtained using standard procedures, for example from seedlings grown from sterile seeds on water agar in darkness in a growth cabinet at a suitable temperature, for example in the range of 22-28 °C, suitably at around 24 °C, for a suitable period of time, for example about 10-60

days. Seeds may be sterilized by immersion in alcohol, for example 70-95% ethanol, for a short period of time, for example 5 seconds to 5 minutes, followed by soaking in a solution containing about 1-3% (suitably 1.5%, v/v) sodium hypochlorite (Javex), or another suitable bleach, for about 15-25 min, suitably about 17-22 min. The bleach

5   solution preferably also contains about 1-10 drops of Tween-20 per 100 mL of solution. Following this sterilization procedure the seeds should be thoroughly rinsed with sterile deionized or distilled water. Some seeds from phytopharmaceutical plants are particularly difficult to sterilize due to a high amount of fungal contamination. In these instances a suitable amount of biostatic agent such as plant preservative mixture

10   (PPM) may be added to the water agar during culture. The amount of the biostatic agent to be used can be determined by culturing the seed or explant on water agar with varying amounts of the biostatic agent to determine the lowest concentration that would be biostatic to the fungal growth and still allow seed germination or explant regeneration.

15

An alternate method for the sterilization of explants may involve the surface sterilization of field or greenhouse-grown plant material. With this method, the tissue would be excised from the intact plant and immediately immersed in sterile water. A similar sterilization protocol would then be followed in which the tissue is rinsed with

20   70-90% alcohol, preferably ethanol, for 30 seconds to 5 minutes, followed by immersion in a bleach solution containing 1-10 drops of Tween-20 per 100 mL for 15-40 minutes. Following this sterilization protocol, the tissue should be thoroughly rinsed in sterile deionized or distilled water and cultured on the induction medium.

25   In one embodiment of the present invention, there is provided a method for the *in vitro* micropropagation of St. John's wort. *De novo* shoot regeneration can be effectively induced by culturing sterile explants of St. John's wort on a medium containing at least one plant growth regulator having cytokinin activity. The plant growth regulator having cytokinin activity may be any suitable natural or synthetic

30   plant growth regulator having cytokinin activity, including TDZ, BAP, zeatin, CPPU and 2-*i*-P. Preferably, the plant growth regulator having cytokinin activity is TDZ. The tissue source for this method can be any suitable sterile tissue from St. John's

wort, for example seeds, stems, petioles, hypocotyl, cotyledon and leaves. Most preferable the tissue is etiolated hypocotyl. The requirement for plant growth regulator having cytokinin activity in the induction medium was satisfied by about 1-15 days, preferably about 6-12 days, more preferably about 8-10 days, exposure to the plant growth regulator. The concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001-25 $\mu mol \cdot L^{-1}$, preferably from about 1.5-20 $\mu mol \cdot L^{-1}$, more preferably about 3-15 $\mu mol \cdot L^{-1}$, even more preferably about 4-10 $\mu mol \cdot L^{-1}$.

Following initial culture on the induction medium, the explants of St. John's wort may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum amount of regenerated shoots/explant. The period of time for subculture will depend on the number of shoots/explant desired and the size restrictions of the culture flask. Optimally, the explants of St. John's wort may be subcultured for about 1-15 days, suitably about 5-12 days, more suitably about 8-10 days. The regenerated shoots may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. At least 90% of the regenerants develop into mature plantlets which are morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants and/or regenerated shoots may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

In the St. John's wort hypocotyl segments cultured on medium in the absence of exogenous growth regulators, a large number of explants were observed to form 1 or 2 adventitious roots. This spontaneous root formation is likely indicative of a high level of endogenous auxin in the etiolated hypocotyls. Supplementation of the culture medium optimized with a plant growth regulator having cytokinin activity suppressed this response. Without wishing to be bound by theory, this effect is potentially a result of an alteration in the auxin-to-cytokinin ratio within the tissues.

The present invention also provides a method for the *in vitro* micropropagation of *Echinacea sp.*, in particular, *Echinacea purpurea*. Sterile explants of *Echinacea* could be cultured on induction medium in the presence of at least one plant growth regulator having cytokinin activity. The plant growth regulator having cytokinin

5 activity may be any suitable natural or synthetic plant growth regulator having cytokinin activity, including TDZ, BAP, zeatin, CPPU and 2-*i*-P. Preferably, the plant growth regulator having cytokinin activity is selected from TDZ and BAP. The tissue source for this method can be any suitable sterile tissue from *Echinacea*, for example seeds, stems, petioles, hypocotyl, cotyledon and leaves. Most preferable the

10 tissue is petiole. The requirement for plant growth regulator in the induction medium was satisfied by about 1-50 days, preferably about 6-40 days, more preferably about 10-35 days, of exposure to the plant growth regulator. If the induction medium also contains an auxin, the time exposure for plant growth regulator may need to be on the higher side of this range, for example up to about 25-40 days. The concentration of

15 plant growth regulator having cytokinin activity may be in the range of from about 0.001-25 μmol·L^{-1}, preferably from about 0.5-20 μmol·L^{-1}, more preferably about 1-15 μmol·L^{-1}. Histological observations revealed that regeneration in petiole cultures of *Echinacea* under the conditions used herein occurred primarily as a result of *de novo* shoot formation from callusing tissue. Other culture conditions may provide

20 regenerated shoots directly. Histological evidence (*supra*) indicates that some somatic embyros were also formed during the culture of *Echinacea* petiole explants on induction medium containing BAP. These somatic embryos may be transferred to basal medium and cultured under suitable conditions to form plantlets.

25 Following initial culture on the induction medium, the explants of *Echinacea* may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum amount of regenerated tissue/explant. The period of time for subculture will depend on the amount of regenerated tissue/explant desired and the size restrictions of the culture flask. Optimally, the explants of

30 *Echinacea* may be subcultured for about 1-15 days, suitably about 5-12 days, more suitably about 8-10 days. The regenerated tissue may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots or

somatic embyros may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. At least 70% of the regenerants develop into mature plantlets which are morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants and/or regenerated tissue may be

5   transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

Indirect morphogenesis is the formation of callus from explants which subsequently results in shoots or somatic embryogenesis (Sharp 1980). Callus

10   formation and cell enlargement in tissues may be indicative of high endogenous auxins (Skoog & Miller, 1957). While not wishing to be limited by theory, the formation of callus at the cut ends of the petioles on the control plates as well as elongation of the explants appears to indicate high levels of endogenous auxins present in petioles of *Echinacea purpurea*. In *Echinacea* petiole cultures, adjacent

15   cells were stimulated to follow different regenerative routes by the same cytokinin. While not wishing to be bound by theory, these data may indicated that the endogenous metabolites in individual cells may have been slightly different, thereby predetermining the fate of the cells in regeneration.

20   The present invention also provides a method for the *in vitro* micropropgation of Huang-qin. Sterile explants of Huang qin could be cultured on induction medium in the presence of at least one plant growth regulator having cytokinin activity. The plant growth regulator having cytokinin activity may be any suitable natural or synthetic plant growth regulator having cytokinin activity, including TDZ, BAP,

25   zeatin, CPPU and 2-*i*-P. Preferably, the plant growth regulator having cytokinin activity is selected from TDZ and BAP. Most preferably, the plant growth regulator having cytokinin activity is TDZ. The tissue source for this method can be any suitable sterile tissue from Huang-qin, for example seeds, stems, petioles, hypocotyl, cotyledon and leaves. Most preferable the tissue is seed, hypocotyl or stem. Most

30   preferably, the tissue is seed. The requirement for plant growth regulator in the induction medium was satisfied by about 1-30 days, preferably about 10-25 days, more preferably about 14-20 days, of exposure to the plant growth regulator. The

concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001-25 $\mu mol \cdot L^{-1}$, preferably about 0.05-20 $\mu mol \cdot L^{-1}$, more preferably from about 1.5-20 $\mu mol \cdot L^{-1}$, most preferably about 2.0-20 $\mu mol \cdot L^{-1}$.

5   Following initial culture on the induction medium, the explants of Huang-qin may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum number of shoots/explant. The period of time for subculture will depend on the number of shoots/explant desired and the size restrictions of the culture flask. Optimally, the explants of Huang-qin may be

10   subcultured for about 1-15 days, suitably about 5-12 days, more suitably about 8-10 days. The regenerated shoots may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. At least 90% of the regenerants develop into mature plantlets which are

15   morphologically similar to seed grown plants raised in the greenhouse. Alternatively, the explants and/or regenerated shoots may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

         It is another embodiment of the present invention to provide a method for the

20   *in vitro* micropropgation of feverfew. Sterile explants of feverfew could be cultured on induction medium in the presence of at least one plant growth regulator having cytokinin activity. The plant growth regulator having cytokinin activity may be any suitable natural or synthetic plant growth regulator having cytokinin activity, including TDZ, BAP, zeatin, CPPU and 2-*i*-P. Preferably, the plant growth regulator

25   having cytokinin activity is TDZ. The tissue source for this method can be any suitable sterile tissue from Huang-qin, for example seeds, stems, petioles, hypocotyl, cotyledon and leaves. Most preferable the tissue is leaf, stem, petiole and hypocotyl. The requirement for plant growth regulator in the induction medium was satisfied by about 1-50 days, preferably about 10-40 days, more preferably about 20-35 days, of

30   exposure to the plant growth regulator. The concentration of plant growth regulator having cytokinin activity may be in the range of from about 0.001-25 $\mu mol \cdot L^{-1}$,

preferably 0.5-20 $\mu mol \cdot L^{-1}$, more preferably from about 1.5-15 $\mu mol \cdot L^{-1}$, most more preferably about 2.0-8 $\mu mol \cdot L^{-1}$.

5   Following initial culture on the induction medium, the explants of feverfew may optionally be transferred to solid basal medium and subcultured for a further period of time to obtain the maximum number of shoots/explant. The period of time for subculture will depend on the number of shoots/explant desired and the size restrictions of the culture flask. Optimally, the explants of feverfew may be subcultured for about 1-15 days, suitably about 5-12 days, more suitably about 8-10

10   days. The regenerated shoots may then be transferred into suitable culture medium to allow roots and plantlets to grow. For example, the shoots may be transferred to culture boxes containing solid basal medium or to flasks containing liquid basal medium. At least 90% of the regenerants develop into mature plantlets which are morphologically similar to seed grown plants raised in the greenhouse. Alternatively,

15   the explants and/or regenerated shoots may be transferred directly from induction medium to solid or basal medium and cultured under conditions to form plantlets.

          The methods developed herein for the *in vitro* micropropagation of phytopharmaceutical plants is ideal for adaptation for use in a bioreactor-type system

20   such as that shown in Figure 1. The bioreactor is a large scale, sterile vessel for growth of plant cells and intact plants in culture media and in a controlled environment. Usually the growth media is re-circulated through the culture vessel by a series of peristaltic pumps and media is aerated to ensure rapid and prolific growth of the cultured tissue. In this way, the growth of the plants or cell cultures can be

25   maintained within the closed system in perpetuity. The bioreactor system can be optimized to produce intact plantlets for whole plant preparations or the media components can be altered for the efficient production of various secondary metabolites that serve as the active ingredients in the phytopharmaceutical plant. The use of the bioreactor system will provide phytopharmaceutical manufacturers with a

30   year-round supply of high quality, consistent plant material.

Experimental Examples:

General Procedures:

5   Statistical analyses:

The design of all experiments was a complete randomized block and treatments consisted of five replications. All the experiments were repeated at least twice and the data were analyzed using SAS Version 6.12 (SAS Inc., 1995).

10   Significant differences between means were assessed by a Student-Neuman-Keulls means separation test at $P \leq 0.05$.

Light microscopic studies:

15   The explants were harvested at weekly intervals over the 4 week incubation period and fixed immediately in formalin:acetic acid:alcohol (FAA: 5:5:90; V/V) and stored at 4 °C. The tissues were dehydrated with a graded ethanol series followed by paraffin embedding. Eight μm thin sections were cut using an ultra microtome (Porter-Blum Ultra microtome MT-1, Ivan Sorvall Inc., Connecticut, USA). The

20   sections were stained (Johnson, 1942) with alcian green and counter stained with safranine and observed under a light microscope (Zeiss, Germany).

Experiment 1: Micropropagation of St. John's wort

25   St. John's wort seeds were sterilized by immersing in a 70% ethanol solution for 5 s, followed by an immersion in a 30% solution of 5.4% sodium hypochlorite (Lilo Products, Hamilton, Ontario) in water with one drop of Tween 20 per 500 mL for 20 min, and a three times rinse in sterile distilled water. Sterile seeds were germinated and maintained on water agar (8 g·L^{-1}) for 16 days in darkness in a growth

30   cabinet at 24 °C. Hypocotyl sections were excised from sterile etiolated seedlings and cultured on a medium containing MS medium (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al 1968), 30 g·L^{-1} sucrose and 3 g·L^{-1} gellan gum (Gelrite,

Schweitzerhall Inc., South Plainfield, NJ, USA). Varying levels (0, 5, 10, 15 and 20 $\mu mol \cdot L^{-1}$) of TDZ and BAP (Sigma Chemical Co., St. Louis, USA) were incorporated into the basal culture medium in a series of experiments. Each experiment consisted of six explants per plate and 20 plates per plant growth regulator treatment. For

5 determination of the optimal duration of exposure of the explants to the medium containing TDZ, five plates of each treatment were subcultured onto MS basal medium at days 3, 6, 9 and 12. All cultures were incubated in a growth cabinet with a 16 hour photoperiod under cool white light @ 40-60 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Regeneration was quantified after 18 and 23 days of culture.

10

Hypocotyls cultured on a medium without exogenous growth regulators produced an average of 0.8 roots/explant while rooting was not observed on explants cultured on medium containing TDZ (Table 1). *De novo* shoot organogenesis was induced on etiolated hypocotyl segments cultured on media supplemented with TDZ

15 after 18-21 days (Figure 1a). Explants which remained on the TDZ medium throughout the treatment period exhibited symptoms of browning and the regenerated shoots appeared stunted and malformed as compared to those which developed on explants transferred to basal medium following a brief exposure to TDZ. Supplementation of the induction medium with TDZ alone resulted in the induction of

20 significantly more regeneration than any other combination of growth in these experiments. The optimal concentration of TDZ for induction of shoot organogenesis on etiolated St. John's wort hypocotyls under the present conditions was 5 $\mu mol \cdot L^{-1}$. The induction of regeneration was effected by the duration of exposure to TDZ. Under the present conditions, the optimal culture period on TDZ-supplemented

25 medium was 9 days with subculture onto basal media for a further 9 day period. Varying the culture conditions may change these optimal values. This optimal protocol produced a mean of 54.0 shoots/explant in 18 days (Table 2). Regenerated shoots, transferred into culture boxes containing the basal medium, formed roots and whole plantlets within 2 months (Figure 2b and c). Similarly, the regenerated shoots

30 formed vigourously growing plantlets in flasks containing liquid basal medium. More than 95% of the regenerants developed into mature plantlets which were morphologically similar to seed grown plants raised in the greenhouse.

The sections were observed under a light microscope to investigate the histological process of adventitious shoot development in St. John's wort hypocotyl cultures grown on induction medium (5 μmol·L^{-1} TDZ). Abundant meristematic

5   zones developed at the hypodermal layers of the hypocotyl as early as 7 days after culture initiation (Figure 3a, arrows). The meristematic zones consisted of cells which were small in size with dense cytoplasm and a prominent nuclei (Figure 3a). After 9 days of culture, these meristematic zones further differentiated and the development progressed toward the epidermis (Figure 3b). In observations made after two weeks

10   of culture, the development of shoot primordia was clearly visible (Figure 3c, arrows). The shoot primordia developed into fully developed shoots with vascular connections between the vascular bundle of the explant and the developing regenerant by day 18 (Figure 3d).

Table 2: Effects of different concentrations of TDZ and duration of culture on TDZ supplemented medium on regeneration of St. John's wort hypocotyl explants. Statistical differences assessed by the Student Newman-Kuells mean separation test.

[TDZ] (μmol·L^{-1})	Day transferred onto basal medium	Number of Shoots/hypocotyl	Number of Roots/hypocotyl
0	0	4.6[e]	0.8[a]
5	6	27.9[c]	0.3[bc]
5	9	54.0[a]	0.0[c]
5	12	42.8[ab]	0.0[c]
10	3	11.5[de]	0.2[bc]
10	6	13.5[de]	0.0[c]
10	9	47.6[ab]	0.0[c]
10	12	46.3[ab]	0.0[c]
15	3	12.5[de]	0.1[bc]
15	6	22.6[cd]	0.0[c]
15	9	47.0[ab]	0.0[c]
15	12	30.2[c]	0.0[c]
20	3	20.7[cd]	0.3[b]
20	9	45.2[ab]	0.0[c]
20	12	39.0[b]	0.0[c]

[abcde] Values within a column with different superscripts are significantly different (P<0.05).

Experiment 2: Micropropagation of *Echinacea purpurea*

Echinacea purpurea achenes were sterilized by immersing in 70% ethanol for 30 sec, soaking in 5.4% sodium hypochloride (Javex) in water with one drop of Tween 20 per 100 ml for 18 min and rinsing three times in sterile deionized water. Due to a high amount of fungal contamination present in the seed coat of *Echinacea purpurea* achenes, PPM was included in the water agar to obtain sterile seedling germination for culture. Sterile seeds were germinated on water agar (8 g·L^{-1}) with 3

ml·L^{-1} plant preservation mixture (PPM) in a growth cabinet in 24 hour darkness at 24°C for 14 days. Different concentrations of PPM were included in with the water agar (1, 2, 3, 4 and 5 ml·L^{-1}) to determine the lowest amount which would be biostatic to fungal growth. A concentration of 3 ml·L^{-1} PPM was found to be the optimal

5   concentration for germination of *Echinacea* seeds under the present conditions. This concentration may vary depending on the culture conditions.

      Germinating seedlings were cultured onto MS medium (Murashige & Skoog, 1962) with B5 vitamins (Gamborg et al 1968), 30 g·L^{-1} sucrose and 3 g·L^{-1} gelrite in

10   Magenta boxes. Petiole explants, 2 cm in length were excised from 4 week old sterile *Echinacea purpurea* plants and cultured onto induction medium comprising MS media supplemented with thidiazuron (TDZ) (0.5, 1, 5 and 10 µmol·L^{-1}) or BAP (1, 2.5, 5, 7.5, 10, 12.5 and 15 µmol·L^{-1}). TDZ was added to the media at concentrations of 0.5 and 1.0 µmol·L^{-1} in combination with indole acetic acid (IAA, an auxin) at

15   concentrations of 5 and 10 µmol·L^{-1}. Explants cultured on media containing TDZ and IAA were subcultured onto MS basal media after 23 days while those cultured on media containing higher concentrations of TDZ were subcultured onto MS basal media after 4 and 8 days. All treatments consisted of 10 plates per treatment and 6 explants per plate. Treatments were incubated in a growth cabinet with a 16 hour

20   photoperiod under cool white light @ 40-60 µmol·m^{-2}·s^{-1}. Regeneration was quantified after 25 and 33 days for all petiole cultures and roots after 33 and 42 days of culture. The resulting regenerants were excised from petioles and subcultured onto MS basal media in test tubes for germination after 32 days of culture.

25   Supplementation of the culture medium with BAP as the sole growth regulating compound induced *de novo* shoot formation at all concentrations (Fig. 4, Table 3). In addition to regeneration, supplementation of the culture medium with BAP was also observed to induce callus formation and elongation of the petioles. When IAA was combined with TDZ or the medium was supplemented with TDZ

30   alone, the formation of *de novo* regenerants was observed (Table 4).

Table 3: Effects of the cytokinin BAP on regeneration of *Echinacea purpurea* petiole explants. Statistical differences assessed by the Student Newman-Kuells mean separation test after 33 days of culture.

BAP Concentration (μmol·L^{-1})	Number of regenerants/petiole
0	0.0^c
1	5.4^{ab}
2.5	8.1^a
5	6.6^{ab}
7.5	5.2^{ab}
10	3.9^b
12.5	5.2^{ab}
15	5.5^{ab}

5   ^{abc} Values within a column with different superscripts are significantly different ($P < 0.05$).

Table 4: Effects of the auxin IAA and TDZ on production of somatic embryos and roots of *Echinacea purpurea* petiole explants. Statistical differences assessed by the Student Newman-Kuells mean separation test after 33 days of culture.

IAA Concentration (μmol·L^{-1})	TDZ Concentration (μmol·L^{-1})	Number of regenerants/petiole	Number of roots/petiole
0	0	0.0^b	0.0^b
0	0.5	3.5^a	0.0^b
0	1.0	3.2^a	0.0^b
5	0	0.1^b	4.4^a
5	0.5	3.3^a	0.0^b
5	1.0	2.4^a	0.0^b
10	0	0.6^b	4.5^a
10	0.5	4.9^a	0.1^b
10	1.0	3.3^a	0.0^b

^{ab} Values within a column with different superscripts are significantly different ($P < 0.05$).

Petiole explants cultured on MS media + 5 μmol·L^{-1} BAP were harvested at 0, 3, 5, 7, 14, 21, 28 and 35 days after culture initiation. Samples were immediately fixed in formalin/glacial acetic acid and 50% ethanol (FAA) mixture (5:5:90 v/v/v). Proper and rapid fixation of the sample was ensured by vacuuming the samples at -20

5   kPa for 10 min. The samples were then dehydrated through a graded tertiary butanol series and embedded in paraffin wax. Transverse 8 μm thin sections were cut using an ultra microtome (Porter-Blum ultra microtome MT-1, Ivan Sorvall Inc., Connecticut, USA) and stained with alciar green and safranine (Jensen, 1962). The sections were observed under a compound light microscope (Zeiss, Germany).

10

Histological observations revealed that regeneration in petiole cultures of *Echinacea* occurred primarily as a result of *de novo* shoot formation from callusing tissue. After 3 to 7 days in culture, the epidermal and subepidermal layers of the petiole explant started to divide (fig. 5a) and formed a compact mass of callus tissue

15   (fig. 5b). This callus tissue consisted of numerous meristomatic zones (fig. 5a - arrows, 5c). The cells of these meristomatic zones were small in size with dense cytoplasm and a prominent nuclei. These meristomatic zones further underwent differentiation and formed a dome shaped shoot meristem by day 21 (fig. 5d - arrows). The shoot meristem developed leaf primordia (fig. 5e,f - arrows) and eventually

20   formed shoot buds after 21 days. A well developed shoot bud consisted of a dome shaped shoot meristem surrounded by a few leaf primordia (fig. 5f). The leaf primordia had well developed trichomes. Vascular elements were observed sporadically dispersed within the callus, mainly near the base of the shoot buds (fig. 5f - arrows).

25

In addition to the shoot organogenesis, there was evidence of somatic embryogenesis in the histological examination of the petiole cultures. Proembryo like structures were observed in the subepidermal layers of the petiole as early as 14 days of culture (fig. 6a - arrows). These proembryos appeared globular in shape,

30   multicellular and were surrounded by a singe outer wall. The proembryos later differentiated into heart shaped somatic embryos (fig. 6b). These somatic embyros may be transferred to basal medium and cultured under conditions to form plantlets.

Experiment 3: Micropropagation of Huang-qin

Seed culture:

Huangqin (*Scutellaria biacalensis*) seeds were surface sterilized by dipping in 95% ethanol for 30s, then immersing in 1.5% sodium hypochlorite containing Tween-20 (2 drops per 100 ml solution) for 18 min and then rinsed 3 times in sterile distilled water. Five seeds were cultured aseptically in each Petri dish containing 25 ml of induction medium, which consisted of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al, 1968), $30g \cdot L^{-1}$ sucrose, $3g \cdot L^{-1}$ gelrite and amended with various concentration of TDZ and 4ml/l PPM (for killing fungus in seeds). The medium was adjusted to pH 5.75 before autoclaving at 121 °C, $1.4 \text{ kg} \cdot \text{cm}^{-2}$, for 20 min. The Petri dishes were sealed with Parafilm and incubated in a growth chamber at $24 \pm 2°C$ with a 16 h photoperiod provided by fluorescent tubes at 30-35 $\text{umol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

Huang-qin seeds began to germinate after 7 days of culture. A few seedlings regenerated shoots from crown after 10 days of culture. On day 14, all germinating seedlings on the medium with TDZ began to develop *de novo* shoots. The seedlings cultured on the medium with TDZ at 2.5 $\mu\text{mol} \cdot L^{-1}$ had an average of 19 shoots per seedling, while the seeds germinated on the medium without TDZ had an average of 2 shoots per seedling. The medium containing 5.0, 7.5 and 10 $\mu\text{mol} \cdot L^{-1}$ also showed shoot formation (Table 5).

Table 5: Effect of TDZ on Induction of Shoot Formation in Huang-qin Seedlings After 14 Days of Culture

TDZ Concentration (μmol·L^{-1})	Number of Shoots/Seedling
0.0	2.32[c]
2.5	19.85[a]
5.0	12.05[b]
7.5	17.40[ab]
10.0	17.07[ab]
20.0	17.25[ab]

[abc] Values within a column with different superscripts are significantly different (P<0.05).

Hypocotyl culture:

Huang-qin seeds were sterilized as described above and aseptically cultured in Petri dishes containing 25 mL of 0.8% water agar with 4 mL/L PPM. Cultures were incubated in the dark at 24 °C for 14 days for germination. Six etiolated hypocotyl segments (about 0.5 cm) were excised from the seedlings and cultured on an induction medium comprising MS salts, B5 vitamins, 3% sucrose, 0.3% gelrite and various concentrations of TDZ (0, 2.5, 5, 7.5 and 10.0 μmol·L^{-1}). Cultures were incubated in a growth chamber at 24 ± 2 °C with a 16 h photoperiod provided by fluorescent tubes at 30-35 μmol·m^{-2}·s^{-1} for 14 days.

Huang-qin hypocotyls on the MS basal medium in the absence of TDZ changed color to light purple after 7 days culture. After 10 days, they began to regenerate one or two adventitious shoots. After 14 and 18 days, shoot length increased quickly but no significant increase in shoot number per explant. Hypocotyls cultured on induction medium with TDZ at 2.5, 5.0, 7.5, and 10 μmol·L^{-1} appeared swollen and developed a green color after 7 days of culture. After 10 days, hypocotyls in these treatments began to form shoots on the swollen tissue. The treatment with TDZ at 7.5 μmol·L^{-1} induced significantly more shoots as compared with the control (Table 6). On day 14 and day 18,

the number of shoots per explant on all the TDZ treatments rapidly increased. All explants on the medium containing TDZ formed shoots with an average of 8-11 shoots per explant while the explants cultured on medium containing no plant growth regulator had an average of 1 shoot per explant (Table 6).

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Table 6: Effect of TDZ on Induction of Shoot Regeneration on Huang-qin Etiolated Hypocotyls

TDZ Concentration (μmol·L^{-1})	Number of Shoots on Day 10	Number of Shoots on Day 14	Number of Shoots on Day 18
0.0	1.03[b]	1.08[b]	1.50[b]
2.5	1.96[b]	9.67[a]	11.06[a]
5.0	1.53[b]	9.39[a]	9.44[a]
7.5	2.40[a]	8.00[a]	8.42[a]
10	2.00[b]	8.17[a]	9.33[a]

[ab] Values within a column with different superscripts are significantly different
10   ($P<0.05$).

Epicotyl culture:

15   For determination of the effect of TDZ on Huang-qin epicotyl tissues, stem segments (about 1.0 cm long) from sterile seedlings grown as described above on MS basal medium for 20 days were excised and culture on induction medium. The concentrations of TDZ tested were 0, 2.5, 5.0, 7.5 10 and 20 μmol·L^{-1}. Induction cultures were incubated in a growth chamber at 24 ± 2 °C with a 16 h photoperiod provided by fluorescent tubes at 30-35 umol·m^{-2}·s^{-1} from 14 days. In all experiments, each treatment
20   had 5 replicates and each experiment was repeated at least twice. Regeneration of shoots was observed after 10 days, 14 days and 18 days of culture and quantified at 14 days of culture. The results for the quantification at 14 days are summarized in Table 7.

Huang-qin stems cultured on the medium containing TDZ began to swell at
25   the edges of cuts and formed callus that appeared green in color by day 7.

Regeneration of shoots was observed after 10 days of culture. On day 14, shoots had formed on all explants exposed to TDZ. The explants on the medium with TDZ had an average of 12-14 shoots per stem segment while explants cultured in the absence of growth regulators had an average of 3 shoots per explant.

5

Table 7: Effect of TDZ on Induction of Shoot Formation in Huang-qin Stem Explants Quantified on Day 14

TDZ Concentration (μmol·L^{-1})	Number of Shoots/Explant
0.0	3.54[c]
2.5	13.61[a]
5.0	14.19[b]
7.5	14.58[ab]
10.0	13.78[ab]
20.0	12.61[ab]

[abc] Values within a column with different superscripts are significantly different

10   ($p < 0.05$).

Experiment 4: Micropropagation of Feverfew

Preparation of the explants:

15

Mature feverfew (*Tanacetum parthenium*) were used for all of the experiments. Seeds were carefully selected for uniformity and surface sterilized by immersion in 70% (v/v) ethanol for 3 min., followed by a 20 min. soak in 1.5% (v/v) sodium hypochlorite in water containing 2 drops of Tween-20 per 100 mL and 5

20   rinses with sterile deionized water. Sterilized seeds were individually cultured in 50 mL glass tubes containing 10 mL of water agar with 3 mL/L of PPM. Seeds were germinated in the dark at 24 °C for the first 7 days and then moved to the light (30-35 μE m^{-2}s^{-1}, 16 h photoperiod) were harvested after 2 months and cultured on a regeneration induction medium.

25

Regeneration induction medium:

Feverfew explants were cultured on an induction medium containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1965) and 30 g/L sucrose with thidiazuron (TDZ) or benzylaminopurine (BAP) at 0, 5, 10, 15, 20, 25 or 50 μmol/L. The pH of the medium was adjusted to 5.75 and 0.3% Gelrite (Scott Laboratories, Carson, USA) was included as the gelling agent prior to autoclaving at 1.4 Kg/cm^2 for 20 min. Regeneration was induced on stem, leaf and shoot explants cultured on an induction medium containing TDZ. The optimal level of TDZ supplementation for induction of regeneration of feverfew under the present conditions was 5 μmol/L. This value may vary depending on the conditions used.

Incubation of the cultures:

After 1 month, regenerated shoot cultures were transferred to a basal medium containing MS salts, B5 vitamins and 3% sucrose for further development. The regenerated shoots formed roots and complete plantlets within 2 months of culture on solid basal medium. As well, prolific regeneration of plantlets was observed in cultures transferred to liquid basal medium. The optimal duration of exposure of feverfew explants to liquid basal medium was assessed in a temporary immersion bioreactor system. In this system, 30 day-old explants with prolific shoot regeneration were transferred to a sterile bioreactor flask. Cultures were incubated in the growth room at 35 μmol·m^{-2}·s^{-1} and liquid basal medium was pumped into the flask and drained from the flask at six hour intervals. The flask environment was aerated with a constant flow of sterile air throughout the incubation period. With this protocol, massive proliferation of feverfew plantlets was achieved in a 30 day period.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for the *in vitro* micropropagation of a phytopharmaceutical plant comprising:

    (a) culturing a sterile explant of the phytopharmaceutical plant on a medium comprising at least one plant growth regulator having cytokinin activity for a period of time to form regenerated tissue; and

    (b) transferring said explant and regenerated tissue to a basal medium and culturing under standard conditions to form plantlets.

2. The method according to claim 1, wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.* and feverfew (*Tanacetum parthenium*) and garlic (*Allium sp.*).

3. The method according to claim 2, wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.* and feverfew (*Tanacetum parthenium*).

4. The method according to claim 2, wherein the sterile explant of a phytopharmaceutical plant is grown on a medium comprising one plant growth regulator having cytokinin activity which is selected from thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thiadiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-(2-chloro-4pyridyl)-N'-phenyl urea) and 2-*i*-P (N6-(2-isopentenyl) adenine or 6-gamma, gamma-dimethylallylamino purine).

5. The method according to claim 4, wherein the plant growth regulator having cytokinin activity is selected from thidiazuron (TDZ) and benzylaminopurine (BAP).

6. The method according to claim 5, wherein the plant growth regulator having cytokinin activity is present at a concentration of about 0.001-$25 \mu mol \cdot L^{-1}$.

7. The method according to claim 5, wherein the period of time is about 1-50 days.

8. The method according to claim 1, wherein the explant is selected from a seed, petiole, hypocotyl, stem, cotyledon and leaf.

9. The method according to claim 1, wherein the phytopharmaceutical plant is St. John's wort.

10. The method according to claim 9, wherein the plant growth regulator having cytokinin activity is thidiazuron.

11. The method according to claim 10, wherein the thidiazuron is present at a concentration of about 0.001-$25 \mu mol \cdot L^{-1}$.

12. The method according to claim 11, wherein the thidiazuron is present at a concentration of about 4-$10 \mu mol \cdot L^{-1}$.

13. The method according to claim 9, wherein the period of time to form regenerated tissue is about 1-15 days.

14. The method according to claim 13, wherein the period of time to form regenerated tissue is about 8-10 days.

15. The method according to claim 9, wherein the explant is etiolated hypocotyl.

16. The method according to claim 1, wherein the phytopharmaceutical plant is *Echinacea sp.*.

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17. The method according to claim 16, wherein the plant growth regulator having cytokinin activity is selected from thidiazuron and benzylaminopurine.

18. The method according to claim 17, wherein the plant growth regulator having cytokinin activity is present at a concentration of about 0.001-25 $\mu mol\cdot L^{-1}$.

19. The method according to claim 18, wherein the plant growth regulator having cytokinin activity is present at a concentration of about 1.0-15 $\mu mol\cdot L^{-1}$.

20. The method according to claim 16, wherein the period of time to form regenerated tissue is about 1-50 days.

21. The method according to claim 20, wherein the period of time to form regenerated tissue is about 10-35 days.

22. The method according to claim 16, wherein the sterile explant is petiole.

23. The method according to claim 1 wherein the phytopharmaceutical plant is Huang qin.

24. The method according to claim 23, wherein the plant growth regulator having cytokinin activity is thidiazuron.

25. The method according to claim 24, wherein the thidiazuron is present at a concentration of about 0.001-25 $\mu mol\cdot L^{-1}$.

26. The method according to claim 25, wherein the thidiazuron is present at a concentration of about 1.5-20 $\mu mol\cdot L^{-1}$.

27. The method according to claim 23, wherein the period of time to form regenerated tissue is about 1-30 days.

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28. The method according to claim 27, wherein the period of time to form regenerated tissue is about 14-20 days.

29. The method according to claim 23, wherein the sterile explant is selected from seeds, hypocotyl and stems.

30. The method according to claim 1, wherein the phytopharmaceutical plant is feverfew.

31. The method according to claim 30, wherein the plant growth regulator having cytokinin activity is thidiazuron.

32. The method according to claim 31, wherein the thidiazuron is present at a concentration of about 0.001-25 $\mu mol \cdot L^{-1}$.

33. The method according to claim 32, wherein the thidiazuron is present at a concentration of about 2.0-8.0 $\mu mol \cdot L^{-1}$.

34. The method according to claim 30, wherein the period of time to form regenerated tissue is about 1-50 days.

35. The method according to claim 34, wherein the period of time to form regenerated tissue is about 20-35 days.

36. The method according to claim 30, wherein the sterile explant is selected from leaf, stem, petiole and hypocotyl.

37. The method for the *in vitro* micropropagation of a phytopharmaceutical plant comprising:

(c)   culturing a sterile explant of the phytopharmaceutical plant on a medium comprising at least one plant growth regulator having cytokinin activity for a period of time to form regenerated tissue;

(d)   transferring said explant and regenerated tissue to a basal medium and subculturing for a further period of time to allow formation of regenerated tissue; and

(e)   transferring said regenerated tissue to a basal medium and culturing under standard conditions to form plantlets.

38.   A method according to claim 37, wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew (*Tanacetum parthenium*) and garlic (*Allium sp.*).

39.   A method according to claim 38, wherein the phytopharmaceutical plant is selected from St. John's wort (*Hypericum perforatum* cv. Anthos), Huang-qin (*Scutellaria baicalensis*), *Echinacea sp.*, feverfew (*Tanacetum parthenium*) and garlic (*Allium sp.*).

40.   A method according to claim 37, wherein the sterile explant of a phytopharmaceutical plant is grown on a medium comprising one plant growth regulator having cytokinin activity which is selected from thidiazuron (TDZ, *N*-phenyl-*N'*-(1,2,3-thiadiazol-yl)urea), benzylaminopurine (BAP), zeatin, CPPU (N-2-chloro-4pyridyl)-N'-phenyl urea) and 2-*i*-P(N6-(2-isopentenyl) adenine or 6-gamma, gamma-dimethylallylamino purine).

41.   A method according to claim 40, wherein the plant growth regulator having cytokinin activity is selected from thidiazuron (TDZ) and benzylaminopurine (BAP).

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42.   A method according to claim 41, wherein the plant growth regulator having cytokinin activity is present at a concentration of about 0.001-25 μmol·L^{-1}.

43.   A method according to claim 41, wherein the period of time is about 1-50 days.

44.   A method according to claim 37, wherein the sterile explant is selected from the seed, petiole, hypocotyl, stem, cotyledon and leaf.

45.   A method according to claim 37, wherein said sterile explant and regenerated tissue are subculturing for about 1-15 days to allow formation of regenerated tissue.
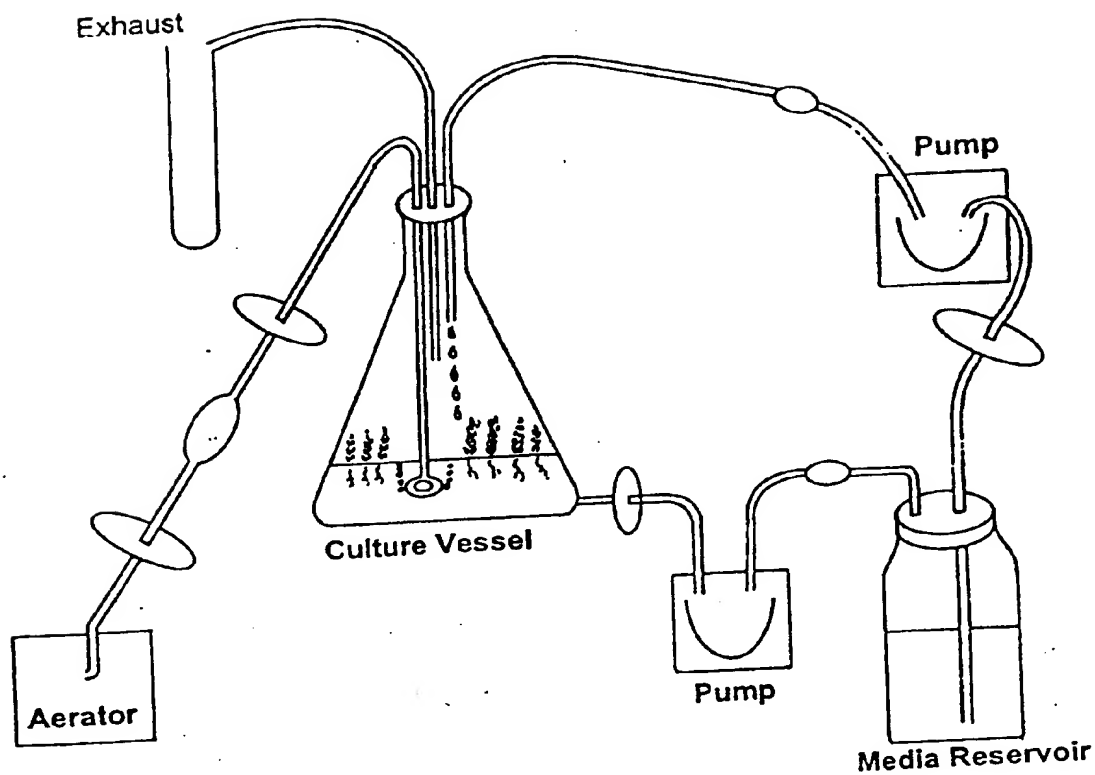
Figure 1

Figure 2(a)
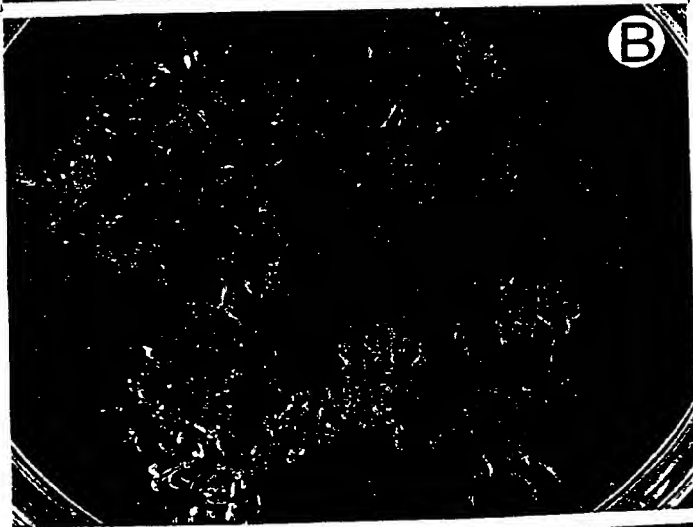
Figure 2(b)
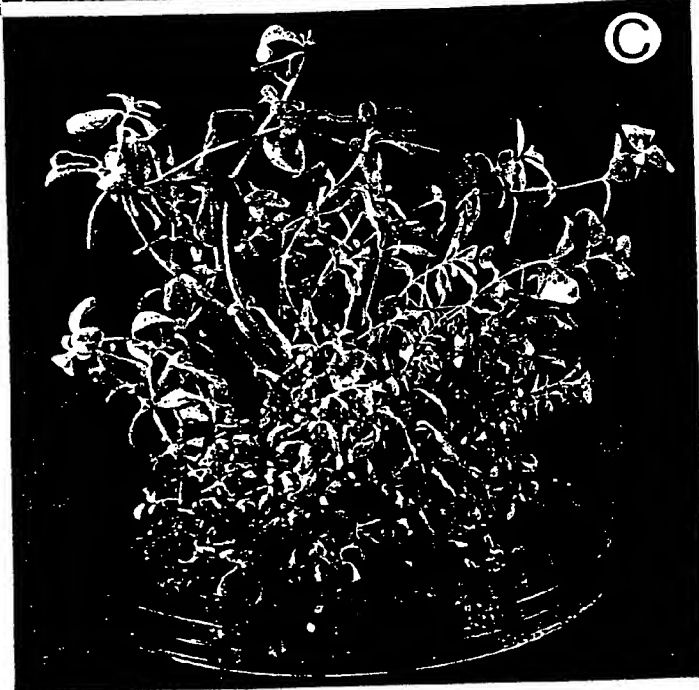
Figure 2(c)

A

Figure 3(a)

Figure 3(b)
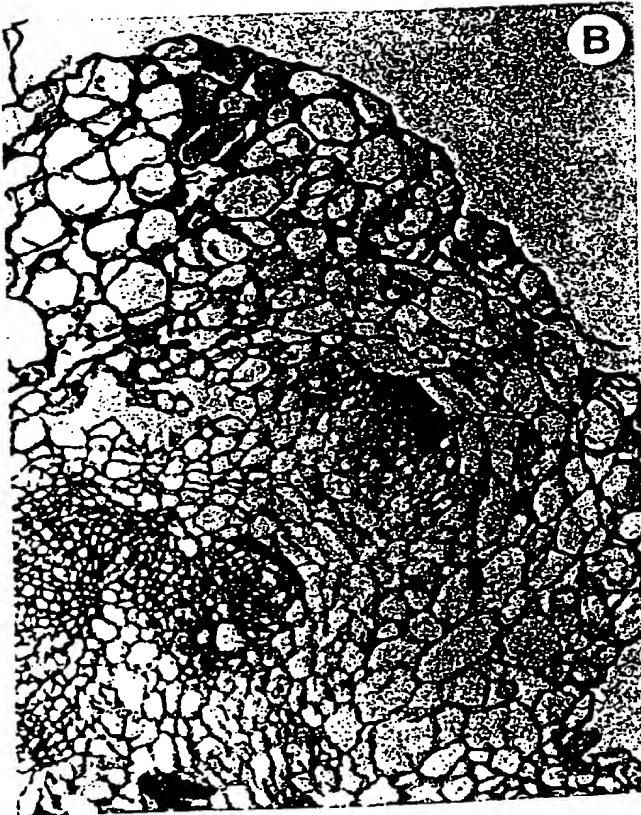
Gowling, Strathy & Henderson
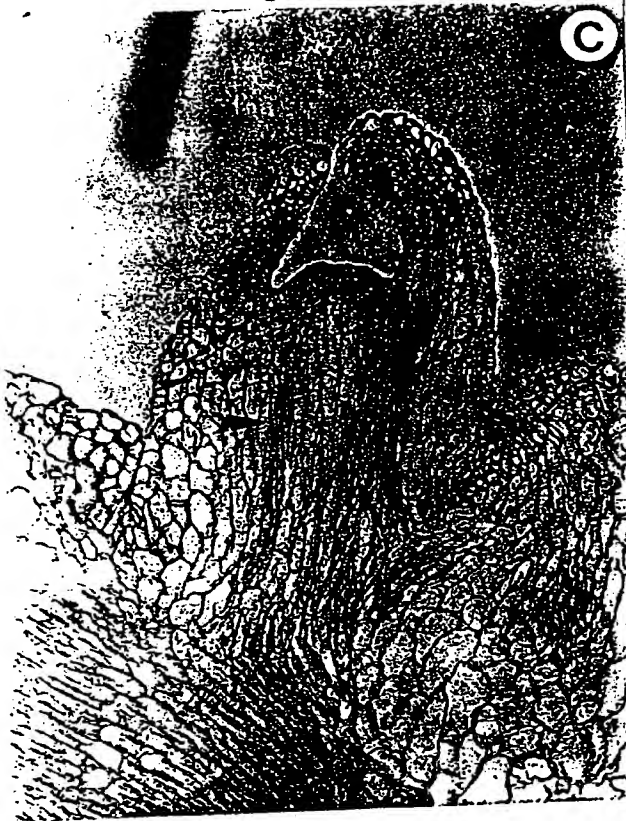
Figure 3 (cont'd)
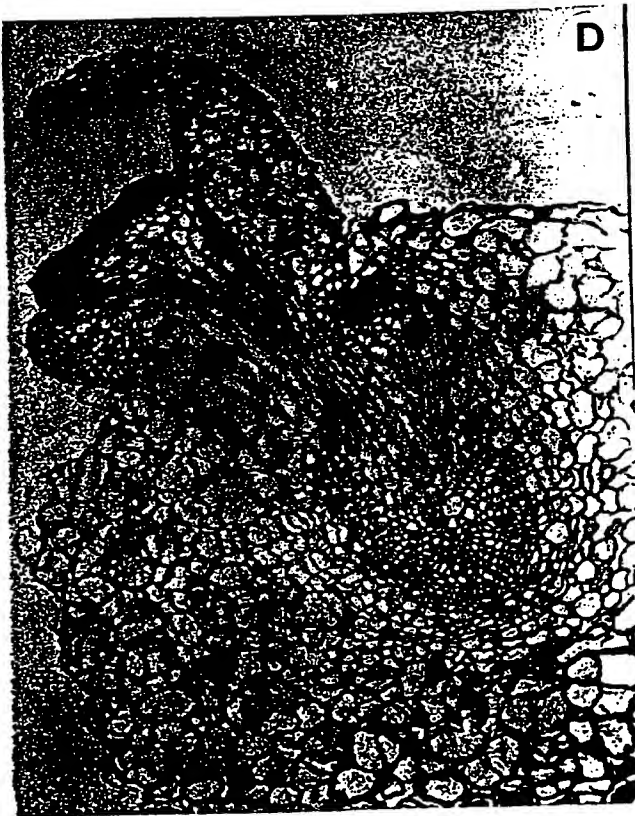
Figure 3(c)

Figure 3(d)

Figure 4(a)

Figure 4(b)

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Figure 4 (cont'd)

Figure 4(c)

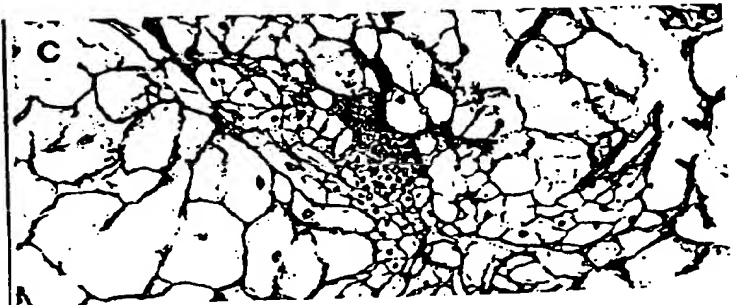Figure 4(d)

Figure 5(a)

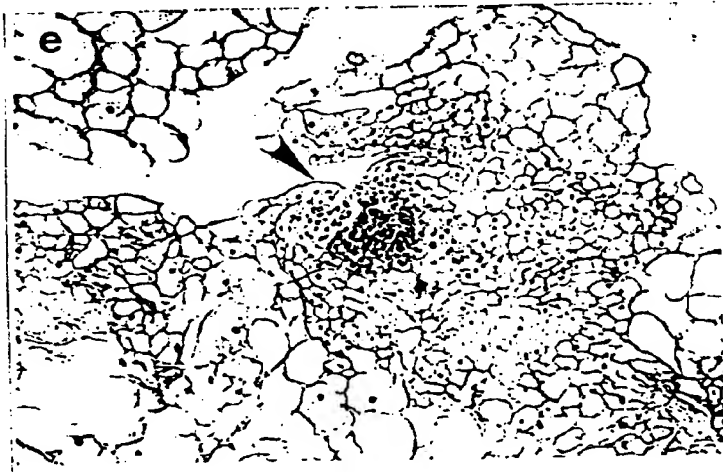Figure 5(b)

Figure 5(c)

Figure 5 (cont'd)
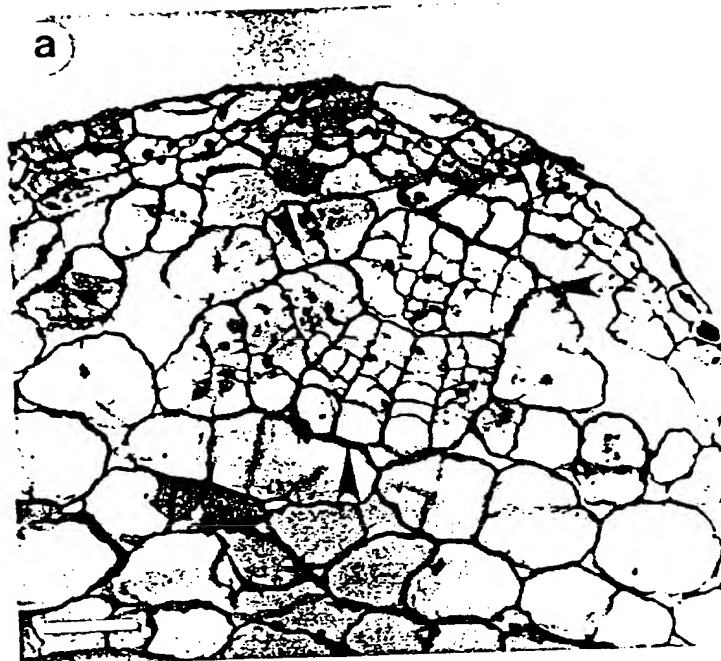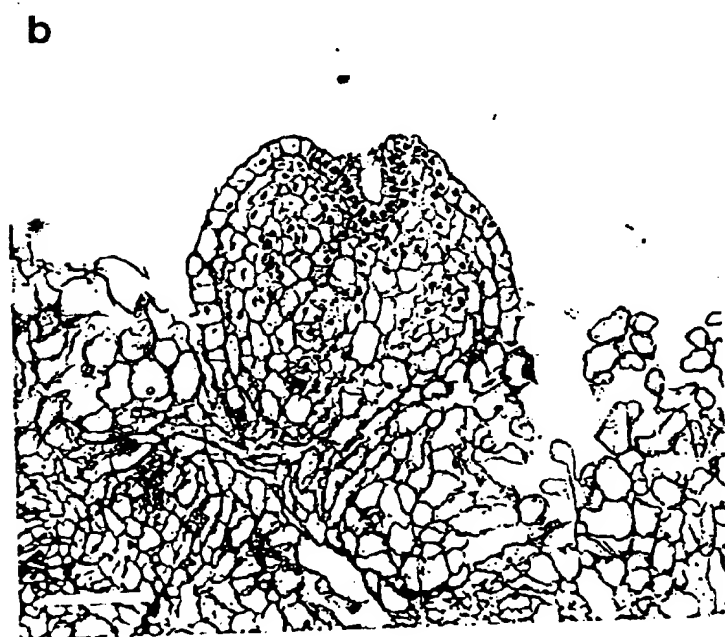

Figure 5(d)

Figure 5(e)

Figure 5(f)

a)

b

Figure 6(a)

Figure 6(b)